trademarked product "Dynabeads™" was added on page 49 to identify the composition and source of the trademarked product Dynabeads™, in accord with the requirements of the official action. The package insert of Dynabeads™ - Cat. #111.03/111.04, obtained from the Internet website of Dynal, is attached in support of the amendment.

Copies of pages 1, 14, 16, 17, 40, 41, and 46, 47, and 49 of the specification, marked to show the changes in the text made by these amendments, are attached. No new matter is introduced by the foregoing amendments.

Support in the Specification for the Amendments and Added Claims:

Claims 2-4, 14, 15, and 18 are canceled; claims 1, 5-13, and 16 are amended, and new claims 19-27 are added.

Support for limiting claim 1 to a method comprising administering an anti-CD20 antibody having B-cell depleting activity, or a fragment thereof having such activity, is found in the specification, for example, at page 4, lines 1-6 and 19-24, which describe B-cell depleting activity of an anti-CD20 antibody. Support for limiting claim 1 to a method comprising administering an anti-CD40L antibody that antagonizes interaction of CD40 and CD40L, or a fragment thereof having such activity, is found in the specification, for example, at page 4, lines 1-6, and page 6, lines 16-22, which describe activities of an anti-CD40L antibody as including antagonizing CD40-CD40L interactions. Support for limiting claims 7 and 8 to a method comprising administering an anti-CD20 or anti-CD40L antibody, respectively, that is a humanized, primatized, or chimeric antibody, is found in the specification, for example, at page 11, line 16, to page 12, line 12. Support for a method wherein the anti-CD20 antibody has at least 10% of the B cell-depleting activity of IDEC-C2B8 as recited in claim 5 is found in the specification, for example, at page 12, lines 29-32.

Support for referring to the trademarked term Rituxan® as IDEC-C2B8, e.g., in claim 6, is found in the specification, for example, at page 1, lines 24-25. Support for reciting an anti-CD40L antibody that is humanized MAb 24-31 as in claim 8 is found in the specification, for example, at page 45, lines 14-15. Support for administering a weekly dose

of said anti-CD20 antibody of 20 to 1000 mg/m^2 as recited in claim 21 is found in the specification, for example, at page 41, lines 11-13. Support for administering a dose of said anti-CD40L antibody of 0.5 to 10 mg/kg body weight as recited in claim 21 is found in the specification, for example, at page 44, lines 21-24. Support for using an anti-CD20 antibody that is radiolabeled with ^{90}Y antibody as recited in claims 22 and 23 is found in the specification, for example, at page 14, lines 1-4. No new matter is introduced.

Regarding the objections to claims 11 and 16:

Claims 11 and 16 are amended to correct the typographical errors identified by the examiner.

Regarding the rejection of the claims under 35 U.S.C. § 103(a):

Claims 1-3, 9-14, and 16-17 were rejected under 35 USC §103(a) as being as obvious in view of Kaminski et al. and/or Anderson et al., in view of Carbone et al., Gruss et al., and Black et al., and in view of standard chemotherapeutic treatments. This rejection is respectfully traversed.

Kaminsky et al. and Anderson et al. disclose administering anti-CD20 antibodies as a treatment for lymphoma; Carbone et al. and Gruss et al. are cited as teaching that B cell lymphomas express CD40 antigens and as suggesting treatment that blocks CD40-CD40L interactions to inhibit proliferation of malignant B cells; and Black et al. is cited as describing humanized anti-CD40L antibodies. Neither the primary nor the secondary references suggest administering both anti-CD20 and anti-CD40L antibodies in combination to treat lymphoma.

The claimed invention is a method for treating lymphoma comprising administering a therapeutically effective amount of an anti-CD20 antibody having B-cell depleting activity, or a fragment thereof having B-cell depleting activity; in combination with an anti-CD40L antibody that antagonizes interaction of CD40 and CD40L, or a fragment thereof that antagonizes interaction of CD40 and CD40L. The invention includes the use of unconjugated and non-radiolabeled anti-CD40L and anti-CD20 antibodies. The inventors have made the

unexpected discovery that administration of an antibody that antagonizes the interaction of CD40 and CD40L unexpectedly and synergistically enhances the anti-lymphoma activity of an anti-CD20 antibody. At the time the application was filed, it was not known that the combination of these two antibodies would interact synergistically to kill lymphoma cells.

Figure 2b is a graph showing % Cytoxicity resulting from incubating IDEC-C2B8 anti-CD20 antibodies or IDEC-131 (humanized MAb 24-31) anti-CD40L antibodies with DHL-4 B lymphoma cells cultured in the presence or absence of 10 μg/ml soluble CD40L (gp39), a concentration of sCD40L that is comparable to the levels of biologically active sCD40L present in the serum of patients with B cell malignancies (see Younes et al., Brit. J. Haematol., 1998, 100(1):135-141; Clodi et al., Brit. J. Haematol., 1998, 103:270-275). Figure 2b shows that the cytoxicity of IDEC-C2B8 anti-CD20 antibodies to B lymphoma cells that are cultured under conditions permitting interaction of CD40 and sCD40L is about 60%; and that the cytoxicity of IDEC-C2B8 antibodies to B lymphoma cells <u>increases to about 90%</u> when the B lymphoma cells are cultured under conditions that prevent interaction of CD40 and CD40L. In contrast, Figure 2b shows that <u>essentially no cytotoxic effect</u> is seen when B lymphoma cells are cultured in the presence of concentrations of IDEC-131 (humanized MAb 24-31) anti-CD40L antibodies that are sufficient to antagonize the interaction of CD40 and CD40L. The disclosed results show that anti-CD40L antibodies that antagonize the interaction of CD40 and CD40L therefore potentiate or enhance the cytoxic effect of anti-CD20 antibodies on B lymphoma cells significantly beyond the level of cytotoxicity expected by adding the cytoxic effects of the two antibodies. The significant enhancement of the cytoxicity of anti-CD20 antibodies to B lymphoma cells that results from blocking the interaction of CD40 and CD40L is also shown in Table I of Example 3 (p. 48), which shows that the induction of apoptosis of B lymphoma cells by anti-CD20 antibodies is about 21% under conditions permitting interaction of CD40 and sCD40L, <u>but increases to about 75% under conditions preventing interaction of CD40 and CD40L</u>. Nothing in the cited prior art suggested that the cytoxic effect of anti-CD20 antibodies on B lymphoma cells would be significantly enhanced by anti-CD40L antibodies that antagonize the interaction of

CD40 and CD40L. The cited references neither disclose nor suggest administering an anti-CD40L antibody and an anti-CD20 antibody in combination to treat a B cell lymphoma. Under U.S. patent law, a suggestion or motivation to combine various teachings of prior art references to obtain the claimed invention, and a reasonable expectation that the combination will operate successfully, must be found in the prior art, not in applicant's disclosure. See M.P.E.P. § 2143, Basic Requirement of a *Prima Facie* Case of Obviousness, citing In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). Even if the prior art could have been taken to suggest administering both antibodies, it would only have amounted to a suggestion to try, and then to observe what happens. Whether the combination would be any more effective than either antibody alone could not have been predicted. "Obvious to try" is not the standard for supporting an obviousness rejection under 35 USC 103. See In re O'Farrell, 7 USPQ2d 1673 (Fed. Cir. 1988). The significant enhancement of the cytotoxicity of anti-CD20 antibodies to B cell lymphoma cells by anti-CD40L antibodies that antagonize the interaction of CD40 with sCD40L, as shown in the disclosed example, could not have been predicted by persons of ordinary skill in the art.

At the time the invention was made, combination therapies were used in treating malignancy; however, they generally involved the combined use of radiotherapy or chemotherapy; with some recent clinical studies reporting the testing of these in combination with a single type of therapeutic monoclonal antibody. Combination therapies with two different antibodies were not described by the prior art. The fact that Rituxan® is the first antibody to be approved for treatment of any B cell malignancy further attests to the uncertainties and unpredictabilities associated with use of antibodies for therapy, and especially treatment of B cell malignancies.

Given the absence of any precedent for the claimed invention in the prior art, given that the prior art references neither disclosed nor suggested using the claimed combination of antibodies to treat B cell lymphoma, there is no basis for the position that one of ordinary skill in the art would have been motivated by the teachings of the cited prior art references to practice the claimed invention with a reasonable expectation of success.

In view of the foregoing, the Applicants respectfully request withdrawal of the rejection of the claims as being obvious under 35 U.S.C. §103(a).

Regarding Provisional Rejection of the Claims for Obviousness-type Double Patenting:

Claims of the application are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims of co-pending U.S. Application No. 09/435,992. The Applicants respectfully request withdrawal of this rejection, because the present claims are directed to treatment of lymphoma, whereas the claims of co-pending U.S. Application No. 09/435,992 are directed to treatment of leukemia.

If the Examiner has any further questions or issues to raise regarding the subject application, it is respectfully requested that he contact the undersigned so that such issues may be addressed expeditiously.

Respectfully submitted,
PILLSBURY WINTHROP LLP

By: [signature]

Robin L. Teskin
Registration No. 35,030

Date: January 2, 2003

1600 Tysons Boulevard
McLean, Virginia 22102
(703) 905-2000
(703) 905-2500

APPENDIX

IN THE CLAIMS:

Claims 2-4, 14, 15, and 18 are canceled, and claims 1, 5-13, and 16 are amended as shown below:

-- 1. (Amended) A method of treating [a B cell malignancy] non-Hodgkin's lymphoma in a subject in need of such treatment comprising administering a therapeutically effective amount of [at least one immunoregulating or immunomodulating antibody that is selected from the group consisting of an anti-CD23, anti-B7, anti-CD40, anti-CD40L and anti-CD4 antibody and at least B cell depleting antibody selected from the group consisting of an anti-CD19, anti-CD20, anti-CD22 and anti-CD37 antibody,]:

(i) an anti-CD20 antibody having B-cell depleting activity, or a fragment thereof having B-cell depleting activity; and

(ii) an anti-CD40L antibody that antagonizes interaction of CD40 and CD40L, or a fragment thereof that antagonizes interaction of CD40 and CD40L;

wherein said antibody administration is effected separately, in combination, and in either order of administration.

2. (canceled)

3. (canceled)

4. (canceled)

5. (Amended) The method of Claim 1 wherein [treatment comprises the administration of an anti-B7 antibody and an] the anti-CD20 antibody is a humanized, primatized, or chimeric anti-CD20 antibody.

6. (Amended) The method of Claim 5 wherein the anti-CD20 antibody [is RITUXAN®] has at least 10% of the B cell-depleting activity of IDEC-C2B8.

7. (Amended) The method of claim [5] 1 wherein the [anti-B7] anti-CD40L antibody is [Primatized] a humanized, primatized, or chimeric anti-CD40L antibody.

8. (Amended) The method of claim 7 wherein the [anti-B7 induces apoptosis of cancer cells] anti-CD40L antibody is humanized MAb 24-31.

9. (Amended) The method of Claim 1 wherein the [immunoregulatory] anti-CD40L antibody is administered after the [B cell depleting] anti-CD20 antibody.

10. (Amended) The method of Claim 1 wherein the [immunoregulatory] anti-CD40L antibody is administered before the [B cell depleting] anti-CD20 antibody.

11. (Amended) The method of Claim 1 wherein the [B cell depleting] anti-CD20 antibody and the [immunoregulatory] anti-CD40L antibody are administered within about a month of each other.

12. (Amended) The method of Claim 1 wherein the [B cell depleting] anti-CD20 antibody and the [immunoregulatory] anti-CD40L antibody are administered within about one week of each other.

13. (Amended) The method of Claim 1 wherein the [B cell depleting] anti-CD20 antibody and the [immunoregulatory] anti-CD40L antibody are administered within about 1 day of each other.

14. (canceled)

15. (canceled)

16. (Amended) The method of Claim 1 wherein [either or both antibody] at least one of the antibodies is attached to a radiolabel.

18. (canceled)

APPENDIX 2

Marked-up pages 1, 14, 16, 17, 40, 41, 46, 47, and 49, showing changes in the text of the specification introduced made by the foregoing amendments, are attached hereto.

TREATMENT OF B CELL MALIGNANCIES USING COMBINATION OF B CELL DEPLETING ANTIBODY AND IMMUNE MODULATING ANTIBODY RELATED APPLICATIONS

Cross Reference to Related Application

This application is a continuation-in-part of US Serial No. 09/435,992 filed November 8, 1999 incorporated by reference in its entirety thereon.

Field of the Invention

The invention relates to a synergistic combination antibody therapy for treatment of B cell malignancies, especially B cell lymphomas and leukemias. This synergistic antibody combination comprises at least one antibody having substantial B cell depleting activity (e.g., an anti-CD19, CD20, CD22 or CD37 antibody) and an antibody that modulates or regulates the immune system, e.g., by modulating B cell/T cell interactions and/or B cell activity, differentiation or proliferation (e.g., anti-B7, anti-CD40, anti-CD23 or anti-CD40L).

Background of Invention

It is known that B cell malignancies, e.g., B cell lymphomas and leukemias may be successfully treated using antibodies specific to B cell antigens that possess B cell depleting activity. Examples of B cell antibodies that have been reported to possess actual or potential application for the treatment of B cell malignancies include antibodies specific to CD20, CD19, CD22, and CD37.

In fact, a chimeric anti-CD20 antibody, RITUXAN® (also known as ~~Rixtimab~~ Rituximab, MabThera®, IDEC-C2B8 and ~~C2B~~ C2B8) is the first FDA approved monoclonal antibody for treatment of cancer (non-Hodgkin's lymphoma) and was developed by IDEC Pharmaceuticals Corporation (see US Patent ~~No's~~ Nos. 5,843,439; 5,776,456; and 5,736,137).

Also, the use anti-CD37 antibodies having B cell depleting activity have been well reported to possess potential for treatment of B cell lymphoma. See e.g., Presr et al., *J. Clin. Oncol.* 7(8): 1027-1038 (August 1989); Grossbard et al., *Blood* 8(4): 863-876 (August 15, 1992).

Further, the use of B cell antibodies specific to CD22 for treatment of B cell malignancies has been reported. For example, an unlabelled antibody that binds

Specific examples of antibodies which bind the CD20 antigen include: "Rituximab" ("RITUXAN®") (US Patent No. 5,736,137, expressly incorporated herein by reference); yttrium-[90]-labeled 2B8 murine antibody "Y2B8" (US Patent No. 5,736,B7, expressly incorporated herein by reference); murine IgG2a "B1" optionally labeled with ^{131}I, "^{131}I B1" ~~1311, "1311 B~~1" antibody (BEXXARTM) (US Patent No. 5,595,721, expressly incorporated herein by reference); murine monoclonal antibody "1F5" (Press *et al. Blood* 69(2):584-591 (1987); and "chimeric 2H7" antibody (US Patent No. 5,677,180, expressly incorporated herein by reference).

Specific examples of antibodies which bind CD22 include LymphocideTM reported by Immuno-medics, now in clinical trials for non-Hodgkin's lymphoma. Examples of antibodies that bind B7 antigen include the B7 antibody reported U.S. Patent 5,885,577, issued to Linsley et al, the anti-B7 antibody reported in U.S. Patent 5,869,050, issued in DeBoer et al, assigned to Chiron Corporation, and the primatized® anti-B7 antibody disclosed in U.S. Patent 6,113,198 to Anderson et al., all of which are incorporated by reference in their entirety.

Specific examples of antibodies that bind CD23 are well known and preferably include the primatized® antibodies specific to human CD23 reported by Reff *et al.,* in U.S. Patent 6,011,138, issued on July 4, 1999, co-assigned to IDEC Pharmaceuticals Corp. and Seikakagu Corporation of Japan; those reported by Bonnefoy *et al.,* No. 96 12741; Rector *et al. J. Immunol.* 55:481-488 (1985); Flores-Rumeo *et al. Science* 241:1038-1046 (1993); Sherr *et al. J. Immunol.,* 142:481-489 (1989); and Pene *et al., PNAS, USA* 85:6820-6824 (1988). Such antibodies are reportedly useful for treatment of allergy, autoimmune diseases, and inflammatory diseases.

The terms "rituximab" or "RITUXAN®" herein refer to the genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen and designated "C2B8" in US Patent No. 5,736,B7, expressly incorporated herein by reference. The antibody is an IgGI kappa immunoglobulin containing murine light and heavy chain variable region sequences and human constant region sequences. Rituximab has a binding affinity for the CD20 antigen of approximately 8.0 nM. ~~8.OnM.~~

An "isolated" antagonist is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or

Other types of lymphoma classifications include immunocytomal Waldenstrom's MALT-type/monocytoid B cell, mantle cell lymphoma B-CLL/SLL, diffuse large B-cell lymphoma, follicular lymphoma, and precursor B-LBL.

As noted B cell malignancies further includes especially leukemias such as ALL-L3 (Burkitt's type leukemia), chronic lymphocytic leukemia (CLL) and monocytic cell leukemias.

The expression "therapeutically effective amount" refers to an amount of the antagonist which is effective for preventing, ameliorating or treating the Ball malignancy disease in question.

The term "immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to suppress or mask the immune system of the mammal being treated herein. This would include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines (see U.S. Pat. No. 4,665,077, the disclosure of which is incorporated herein by reference), azathioprine; cyclophosphamide; bromocryptine; danazol; dapsone; glutaraldehyde (which masks the MHC antigens, as described in U.S. Pat. No. 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as glucocorticosteroids, *e.g.*, prednisone, methylprednisolone, and dexamethasone; cytokine or cytokine receptor antagonists including anti-interferon-α, β- or δ-antibodies, anti-tumor necrosis factor-α antibodies, anti-tumor necrosis factor-β antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CDl la and anti-CD18 antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a antibodies; soluble peptide containing a LFA-3 binding domain (WO 90/08187 published 7/26/90), streptolanase; TGF-β; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (Cohen *et al., U.S.* Pat. No. 5,114,721); T-cell receptor fragments (Offner *et al., Science,* 251: 430-432 (1991); WO 90/11294; laneway, *Nature,* 341: 482 (1989); and WO 91/01133); and T cell receptor antibodies (EP 340,109) such as T10B9.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended
At-211, I-131, I-125, Y-90, Re-186, Re-188, Sm-153,
to include radioactive isotopes *(e.* g. ~~At211 I131 I125~~ Y9o Re 186 Re lg8 sM153

Bi-212, P-32,
~~Bi212 p32~~ and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamime nitrogen mustards such as chiorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,

or in macroemulsions. Such techniques are disclosed in *Remington 's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained release preparations include semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, noir degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

V. Treatment with the B Cell Depleting Antibody and Immunoregulatory Antibody

A composition comprising B cell depleting antibody and/or an immunoregulatory antibody will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular B cell malignancy or disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disease or disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The therapeutically effective amount of the antagonist to be administered will be governed by such considerations.

As noted previously, the B cell depleting antibody and the immunoregulatory antibody may be in the same or in different formulations. These antagonist formulations can be administered separately or concurrently, and in either order. Preferably, the B cell depleting antibody specific to the B cell antigen target, *e.g.*, CD20, CD19, CD22, or CD37 ~~or CD22,~~ will be administered separately from the immunoregulatory antibody, *e.g.*, an anti-CD40L antibody, anti-CD40 antibody, or anti-B7 antibody. Preferably, the CD40L antibody will be the humanized anti-CD40L

antibody disclosed in U.S. Patent 6,001,358 and the anti-B7 antibody the primatized antibody disclosed in US Patent 6,113,898. As noted, this antibody has recently been show to possess apoptotic activity. Also the preferred CD40L antibody has been shown to have efficacy in treatment of both T and B cell autoimmune diseases. Also, unlike another humanized anti-CD40L antibody *(5c8)* reported by Biogen, this antibody is not known to cause any adverse toxicity.

As a general proposition, the therapeutically effective amount of an antibody administered parenterally per dose will typically be in the range of about 0.1 to 500 mg/kg of patient body weight per day, with the typical initial range of antagonist used being in the range of about 2 to 100 mg/kg.

The preferred B cell depleting antibody is RITUXAN®. Suitable dosages for such antibody are, for example, in the range from about 20mg/m2 to about 1000mg/m2. The dosage of the antibody may be the same or different from that presently recommended for RITUXAN® for the treatment of non-Hodgkin's lymphoma. For example, one may administer to the patient one or more doses of substantially less than 375mg/m2 of the antibody, *e.g.* where the dose is in the range from about 20mg/m^2 to about 250mg/m^2, for example from about 50mg/m^2 to about 200mg/m^2.

Moreover, one may administer one or more initial doses) of the antibody followed by one or more subsequent dose(s), wherein the mg/m^2 dose of the antibody in the subsequent doses) exceeds the mg/m^2 dose of the antibody in the initial dose(s). For example, the initial dose may be in the range from about 20mg/m^2 to about 250mg/m^2 *(e.g.* from about 50mg/m^2 to about 200mg/m^2) and the subsequent dose may be in the range from about 250mg/m^2 to about 1000mg/m^2.

As noted above, however, these suggested amounts of both immunoregulatory and B cell depleting antibody are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated above. For example, relatively higher doses may be needed initially for the treatment of ongoing and acute diseases. To obtain the most efficacious results, depending on the particular B cell malignancy, the antagonist is administered as close to the first sign, diagnosis, appearance, or occurrence of the disease or disorder as possible or during remissions of the disease or disorder.

The antibodies are administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local

[1- (average RFU of test sample ÷ Average RFU of control cells)] x 100%. Titration curve of ADM cytotoxicity was established and minimal concentrations of the drug for cytotoxicity was selected for subsequent assays.

The results, as displayed in Fig. 1, shows cell cytotoxicity of DHL-4 cells cultured for 5 days after being exposed to ADM (2×10^{-7} M and 4×10^{-8} M of ADM) for 4 hours prior to culture. Cells were washed once after exposure and cultured in growth medium for 5 days and cytotoxicity determined by Alamar Blue dye-uptake assay, as described above. Additionally, the DHL-4 cells were characterized for the membrane expression of selected CD molecules by flow cytometry. DHL-4 cells have been found to express CD 19, CD20, CD40 molecules, but no expression of CD40L was detected.

Example 2

Anti -CD40L antibody overrides CD40L mediated resistance to killing by to killing, by adriamy in of -lymphoma cells

Fig. 2A shows the effect of an anti-CD40L antibody (IDEC-131) on CD40L-CD40 mediated resistance of DHL-4 cells to cell death induced by ADM. DHL-4 cells (0.5×10^{6} cells/ml) were incubated in the presence of 10 μg/ml of soluble CD40L (sCD40L, P. A. Brams, E. A. Padlan, K. Hariharan, K. Slater, J. Leonard, R. Noelle, and R. Newman, "A humanized anti-human CD 154 monoclonal antibody blocks CD 154-CD40 mediated human B cell activation," *(manuscript submitted)*) for 1 hour at 37°C. After 1 hour of incubation, low concentrations of ADM (2×10^{-7} M - 4×10^{-8} M) were added and incubated for another 4 hours in the presence or absence of ~~CD40L~~ sCD40L (10 μg/ml). Following exposure to ADM, cells were washed and resuspended in growth medium at 0.5×10^{6} cells/ml concentration, and 100 μl of cell suspension added to each well of 96-well flat bottom plate, in duplicate, with or without sCD40L. sCD40L (10 μg/ml) was added to cultures that have been continuously exposed to sCD40L during ADM treatment and to cultures that had no sCD40L during ADM exposure. In addition, IDEC-131 at 10 μg/ml was added to cultures to determine its effect on DHL-4 cells incubated with sCD40L and ADM. After 5 days, the cytotoxicity was measured by Alamar Blue dye-uptake assay, as described.

Data show that sCD40L prolonged survival of DHL-4 cells after ADM treatment, whereas, as expected, increased cytotoxicity was observed in cells that were exposed to ADM in the absence of sCD40L. Furthermore, addition of anti-CD40L antibody (IDEC-131) reversed CD40L mediated cell survival, leading to increase in cell cytotoxicity (**Fig. 2A**).

The addition of IDEC-131 alone had no effect on DHL-4 cells treated with sCD40L, which indicates that the antibody, by itself, does not have any direct inhibitory or cytotoxic activities on DHL-4 cells (**Fig. 2B**). DHL-4 cells pre-incubated with and without sCD40L were cultured in the presence of different concentrations of IDEC-131, RITUXAN®, the anti-CD20 antibody, and CE9.1, ~~and~~ an anti-CD4 antibody ~~antibodies~~ (Anderson et al., *Clin. Immunol. & Immunopathol.* 84: 73-84 (1997)). After 5 days, the cytotoxicity/proliferation of DHL-4 cells was determined by Alamar Blue assay, as described above. **Fig. 2B** shows no effect on the proliferation or the cytotoxicity of DHL-4 cells by IDEC-131, whereas RITUXAN® ~~RITUNAN®~~, as expected, inhibited cell proliferation and induced cytotoxicity. No effect was seen in the DHL-4 cells cultured with anti-CD4 antibodies.

Example 3

CD40L-CD40 signaling prevents apoptosis of B-lymphoma cells by anti-CD20 antibody, RITUXAN®

The effect of CD40L-CD40 mediated signaling on anti-CD20 antibody induced apoptosis of B-lymphoma cells was determined using an *in vitro* system involving DHL-4 cells and the surface cross-linking of RITUXAN®. DHL-4 cells (0.5 to 1 x 10^6 cells/ml) were cultured with sCD40L (10 μg/ml) at 37° C. After overnight culture, cells were harvested and incubated with 10 μg/ml of RITUXAN® or the control antibody (CE9.1; an anti-CD4 antibody) with or without sCD40L (10 μg/ml) on ice. After 1 hour of incubation, cells were centrifuged to remove unbound antibodies, and resuspended at 1 x 10^6 cells/ml in growth medium (5% FCS-RPMI) and cultured in tissue culture tubes. The cells surface bound antibodies were cross-linked by spiking $F(ab')_2$ fragments of goat anti-human Ig-Fcγ specific antibodies at 15 μg/ml, and the cultures were incubated at 37° C. until assayed for apoptosis. Apoptosis was detected using a flow cytometry caspase-3 assay. Cultured cells were harvested at 4 and 24 hours, washed and fixed at 4° C. using Cytofix

Culture Conditions	% Apoptosis (IVHF)[a]	
	4 Hours	24 Hours
Cells + anti-hu.IgG.F(ab')$_2$	6.09 (12.27)	1.85 (17.27)

[a] Percent positive cells with caspase-3 activity and its mean fluorescent intensity in log scale.

Example 4

Effect of IDEC-131 on the survival of chronic lymphocytic leukemia (CLL) cells

To determine the effect of IDEC-131 on the growth and survival of B-CLL cells *in vitro*, B-CLL cells were cultured with and without IDEC-131 in the presence of CD40L *in vitro*. Peripheral blood mononuclear cells (PBMC) were isolated from a CLL patient's blood using a Ficoll-Hypaque gradient centrifugation. Viability was determined by Trypan blue dye exclusion and was >98%. Flow cytometric analysis revealed that >70% of the lymphocytes were CD 19$^+$/CD20$^+$. CLL cells (PBMC) were cultured in CLL growth medium (e.g., RPMI-1640 medium supplemented with 5% FCS or 2% of autologous donor plasma, supplemented with 2 mM L-Glutamine and 100 U/ml Penicillin-Streptomycin). In addition, for some experiments, CD19$^+$ B-cells were purified using CD19$^+$ Dynabeads™ (uniform, 4.5 μm, magnetizable polystyrene beads coated with a primary antibody for CD4) as per manufacture's instructions (Dynal, Oslo, Norway, Cat. #111.03/111.04) and cultured as above. CLL or purified B-CLL cells cultured in growth medium mostly under went spontaneous apoptotic cell death. However, culturing these cells in the presence of sCD40L extended their viability in cultures. **Table II** indicates the cell viability of CD 19$^+$ B-CLL cells grown in the presence or absence of sCD40L (5 μg/ml) at different time points and indicates the longer survival of CLL cells. B-CLL cells from Patient #1 cultured with sCD40L had ≥ 60% viability for greater than 2 weeks, whereas cells grown in the absence of sCD40L had less than 10% viability.

Table II:

Survival of B-CLL cells in the presence of sCD40L

B-CLL Sample	Time (Hours)	%Viability[a]	
		(-) CD40L	(+) CD40L
Patient #1	0	≥90	≥90
	48	88	90
	96	46	77

P sitive Is lati n f CD4 Cells - Package Insert

This document is a copy of the instructions following the product.

Prod. No. 113.03
113.04
Printed: 0201
Rev. no.: 002

CD4 Positive Isolation Kit
(T-helper/inducer cells)

For the rapid immunomagnetic isolation of CD4 positive human cells with Dynabeads® CD4 for use with DETACHaBEAD®, followed by bead detachment with DETACHaBEAD CD4.
For research use only.

INTRODUCTION
Immunomagnetic cell isolation using Dynabeads CD4 for use with DETACHaBEAD, provides a fast and reliable method for capture of CD4+ T cells. The cells can be captured directly from whole blood, buffy coat, single cell suspensions of tissue or suspensions of mononuclear cells as obtained by density gradient centrifugation. During a short incubation period, the CD4+ T cells bind t the Dynabeads, and subsequently the rosetted cells can be washed and isolated by using a magnet (Dynal MPC®). Captured cells are released from the Dynabeads with DETACHaBEAD and are pure (>99%), show high viability (>95%) and may be used in several research applications.

PRODUCT DESCRIPTION
Dynabeads CD4 for use with DETACHaBEAD are uniform, magnetizable polystyrene beads (4.5 µm) coated with a primary monoclonal antibody (mAb) specific for the CD4 membrane antigen which is predominantly expressed on the helper/inducer subset of human T cells.

The Dynabeads are supplied as a suspension containing 1.4×10^8 beads/ml in phosphate buffered saline (PBS) pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (NaN_3).

DETACHaBEAD CD4 is a polyclonal anti-Fab antibody specific for the CD4 antibody on the Dynabeads. When DETACHaBEAD is added to the bead-bound cells it competes with antibody/antigen binding at the cell surface and releases the antibody and bead from the cell. The target CD4 cells are left viable, unstimulated and without antibody on their surface (1,2,3). The isolated CD4+ cells can then be used in any downstream application.

Material supplied
Prod. No 113.03 contains 2 ml Dynabeads CD4 for use with DETACHaBEAD, plus 1 ml DETACHaBEAD CD4. This kit will isolate on average 6×10^7 CD4 cells from 2×10^8 MNC depending upon CD4 cell number in the starting sample.
Prod. No 113.04 contains 10 ml Dynabeads CD4 for use with DETACHaBEAD, plus 5 ml DETACHaBEAD CD4. This kit will isolate on average 3×10^8 CD4 cells from 1×10^9 MNC depending upon CD4 cell number in the starting sample.

Additional materials required

- Magnetic separation device. For all work with Dynabeads, Dynal Biotech recommends the use of a Dynal magnet (Magnetic Particle Concentrator, Dynal MPC®).
- Apparatus allowing both tilting and rotation of test tubes (e.g. a Dynal sample mixer).
- Blood collecting tubes containing anticoagulant.
- Test tubes, glassware, pipettes.

Monoclonal antibody:
The mouse IgM mAb employed (66.1) recognizes the 59 kDa cell membrane glycoprotein, CD4 antigen. The epitope recognized by the 66.1 mAb resides in the same domain of the CD4 molecule as the epitope recognized by mAbs OKT4a and Leu3a. The CD4 antigen is a receptor for major histocompatibility complex (MHC) class I molecules and HIV. CD4 is expressed on approximately 60% of peripheral blood lymphocytes in healthy individuals.

INSTRUCTIONS FOR USE

A) Dynabeads washing procedure
The Dynabeads CD4 for use with DETACHaBEAD should be washed before use. The washing procedure is facilitated by the use f

a magnetic device (Dynal MPC).

1. Resuspend the Dynabeads in the vial.
2. Transfer the desired amount of Dynabeads to a test tube (use 72 µl/ml MNC or buffy coat r 36 µl beads/ml whole blood). For preparation f MNC or buffy coat - see below.
3. Add 1 ml of PBS/2% FCS and resuspend well.
4. Place the test tube in the magnet (Dynal MPC) for 30 secs and pipette off the supernatant.
5. Resuspend the washed Dynabeads in a volume of PBS/2% FCS equal t that originally pipetted from the vial.

N TE:
During incubation and separation procedures it is important to keep the cell suspension and buffers cold (2-8°C) to prevent attachment of phagocytic cells to Dynabeads.

Separation of CD4+ cells
CD4+ cells can be captured from whole blood, buffy coat or suspensions of mononuclear cells. For efficient capture of cells and subsequent release, always use Dynabeads: CD4 cell ratio between 4 and 10 beads per CD4 cell. The concentration of Dynabeads in the mixing volume is also critical. To capture 95% of target cells, bead concentration must always be $^{3}1 \times 10^7$ beads/ml sample (72 µl).

B) Preparing sample:

MNC: Resuspend MNC at 0.5×10^7 total cells/ml in PBS/2% FCS. For each ml sample processed, use 1×10^7 beads (72 µl).

Buffy coat: Dilute the buffy coat in PBS/2% FCS. For each ml of buffy coat, add 2 ml PBS/2% FCS. Use 1×10^7 beads (72 µl)/ml diluted buffy coat.

Whole blood: Use 0.5×10^7 beads (36 µl)/ml. This is due to the low target cell number in a whole blood sample but it must be noted that this lower bead concentration will reduce cell capture efficiency.
If the CD4+ cell number is unknown or very variable, the cells should be guarantified in a flow cytometer before rosetting.

C) Positive isolation of CD4+ T cells from mononuclear cells (MNC)

1. Prepare the MNC as decribed (B) and cool it to 2-8°C
2. Add the prepared sample to the pre-washed Dynabeads (72 µl beads/ml MNC).
3. Incubate for 20 minutes at 2-8°C on an apparatus that provides both gentle tilting and rotation.
4. Isolate the rosetted CD4+ T cells by placing the test tube in the Dynal MPC for 2-3 minutes.
5. Discard the supernatant while the rosetted cells are attached to the wall of the test tube by the MPC.
6. Wash the rosetted cells 4-5 times in PBS/2% FCS.
7. Resuspend the rosettes in 100 µl RPMI 1640/1% FCS.

D) Positive isolation of CD4+ T cells from buffy coat

1. Prepare the buffy coat as described (B) and cool it to 2-8°C.
2. Add the prepared sample to the pre-washed Dynabeads. (72 µl beads/ml buffy coat).
3. Incubate for 20 minutes at 2-8°C on an apparatus that provides both gentle tilting and rotation.
4. Isolate the rosetted CD4+ T cells by placing the test tube in the Dynal MPC for 2-3 minutes.
5. Discard the supernatant while the rosetted cells are attached to the test tube wall by the MPC.
6. Wash the rosetted cells 4-5 times in PBS/2% FCS.
7. Resuspend the rosettes in 100 µl RPMI 1640/1% FCS for each ml sample processed. For samples less than 1 ml also use 100 µl RPMI 1640/1% FCS.

E) Positive isolation of CD4+ T cells from whole blood.

1. Add the whole blood to the pre-washed Dynabeads. (36 µl beads/ml blood).
2. Mix well by inverting the tube 2-3 times.
3. Incubate for 30 minutes at 2-8°C on an apparatus that provides both gentle tilting and rotation.
4. Isolate the rosetted CD4+ T cells by placing the test tube in the Dynal MPC for 2-3 minutes.
5. Discard the supernatant while the rosetted cells are attached to the test tube wall by the MPC.
6. Wash the rosetted cells 4-5 times in PBS/2% FCS.
7. Resuspend the rosettes in 100 µl RPMI 1640/1% FCS for each 2 ml sample processed (1×10^7 beads, 72 µl). For samples less than 1 ml also use 100 µl RPMI 1640/1% FCS.

F) Detachment

1. Add 10 µl DETACHaBEAD per 100 µl cell suspension prepared as above.
2. Incubate for 45-60 minutes at room temperature with gentle mixing.
3. Place the tube in a Dynal MPC for 2 minutes.
4. Transfer the supernatant (released cells) to a fresh tube.
5. To obtain residual cells, wash the beads 2-3 times in 500 µl RPMI 1640/1% FCS and collect the supernatant.
6. Wash detached cells thoroughly by resuspending the cells in a total of 10 ml and centrifuge for 6-8 minutes at 300-500 g to remove DETACHaBEAD.
7. Resuspend the cells in RPMI 1640/1% FCS or other media and use in downstream application.
8. The isolated cells are pure (>99%), viable (>95%) and are free from antibody bound to the surface.

Buffers and solutions
Isotonic Phosphate Buffered Saline (PBS) pH 7.2 - 7.6.

Dynal Biotech recommends the following PBS, pH 7.4:

$NaH_2PO_4 \times H_2O$ 0.16 g

$Na_2HPO_4 \times 2\ H_2O$ 0.98 g

NaCl 8.10 g
Distilled water to 1 litre

PBS/2% FCS:
Add 2% fetal calf serum (final concentration) to PBS.

0.02% NaN_3 may be added as a preservative if needed.

RPMI 1640/1% FCS:
Add 1% fetal calf serum (final concentration) to RPMI 1640.
All reagents should be analytical grade. The fetal calf serum should be heat inactivated for 30 minutes at 56°C and tested for cytotoxicity before use.

Physical characteristics of Dynabeads:

Diameter: 4.5 µm ± 0.2 µm

Density: approx. 1.5 g/cm^3

Magnetic mass susceptibility: 16 ± 3 x 10^{-5} m^3/kg

Geometric surface area: 6 x 10^{-4} m^2/10^7 Dynabeads (0.9 m^2/g)

REFERENCES

1. Human endothelial cell costimulation of T cell IFN-g production. Briscoe DM et al. 1997 J Immunol 159:3247-3256 (Ref. No. 2231)
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3. adhesion molecules. Friedl P et al. 1995 J Immunol 154:4973-4985 (Ref. No. 1649)
4. Differential requirement of protein tyrosine kinases and protein kinase C in the regulation of T cell locomotion in 3-D collagen matrices. Entschladen F et al. 1997 J Immunol 159:3203-3210 (Ref. No. 2230)

STORAGE AND STABILITY
Dynabeads CD4 for use with DETACHaBEAD and DETACHaBEAD CD4 are stable, when stored unopened at 2-8°C until the expiry date stated on the label. The vial should be stored upright to keep the Dynabeads in liquid suspension. This product Dynabeads contains 0.02% sodium azide as a preservative. This may be cytotoxic and should be carefully removed by washing before use.

NOTE:
The Dynabeads product must be maintained in liquid during storage and during all handling steps, as drying of the Dynabeads will result in reduced performance.

PRECAUTIONS
Resuspend Dynabeads well before use to obtain a homogenous dispersion of beads in solution. Dynabeads should be washed in PBS/BSA or similar.
Precautions should be taken to prevent bacterial contamination of the opened vials.
Preservatives should be carefully removed before use by washing.

WARNING AND LIMITATIONS
DYNABEADS CD4 FOR USE WITH DETACHaBEAD AND DETACHaBEAD CD4 IS FOR RESEARCH USE ONLY.
The product is not for use in human diagnostic or therapeutic procedures.
Preservatives such as sodium azide are toxic if ingested.
Av id pipetting by mouth!
Sodium azide may react with lead and copper plumbing to form highly explosive metal

azides. When disposing through plumbing drains, flush with large volumes of water to prevent azide buildup.

WARRANTY
The products are warranted t the original purchaser only to conform to the quantity and contents stated on the vial and outer labels for the duration of the stated shelf life. Dynal Biotech's obligation and the purchaser's exclusive remedy under this warranty is limited either t replacement, at Dynal Biotech's expense, of any products which shall be defective in manufacture, and which shall be returned to Dynal Biotech, transportation prepaid, or at Dynal Biotech's option, refund of the purchase price. Claims for merchandise damaged in transit must be submitted to the carrier.
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